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In-vitro Metabolism of a Novel Monocrotophos Derivative by Rat and Japanese Quail

Mohd K. J. Siddiqui

Industrial Toxicology Research Centre, Mahatma Gandi Marg, Lucknow, India

& Colin H. Walker*

School of Animal & Microbial Sciences, University of Reading, PO Box 228, Reading RG6 6AJ, UK

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Abstract: NADPH-dependent inhibition of hepatic microsomal carboxylesterase by a derivative of monocrotophos (coded as RPR-5) was studied in rat and Japanese quail as a measure of monooxygenase-catalysed activation of RPR-5. There was NADPH-dependent inhibition of hepatic microsomal α-naphthyl acetate esterase (carboxylesterase) both in rat and quail, indicating monooxygenase-catalysed formation of an oxon that subsequently phosphorylated α-NaE. The pattern of in-vitro metabolism of ^{14}C-labelled RPR-5 by 11 000*g* supernatant (11-S), microsomes and 105 000*g* supernatant (105-S) fractions of rat and quail livers suggested the involvement of microsomal monooxygenases and carboxylesterases. A radiolabelled metabolite (M2) was tentatively identified as an acid produced by carboxyl esterase attack. In rat, metabolism by microsomal and cytosolic (105-S) carboxylesterases appeared to predominate with relatively little oxidative metabolism. In quail, putative microsomal carboxylesterase hydrolysis of RPR-5 was much lower than in the rat with almost neglible hydrolysis by cytosolic fractions. Also, production of M_2 by quail microsomes was substantially reduced after addition of NADPH, suggesting inhibition of a carboxyl esterase by the oxon of RPR-5. Differences in this detoxification of RPR-5 between rat and quail may be an important factor in determining selective toxicity and the results underline the importance of relating metabolism to toxicity when selecting animal models for toxicity testing.

Key words: metabolism, microsomes, organophosphorus insecticide, rat, quail

1 INTRODUCTION

The environmental persistence, the development of resistance to pesticides by target organisms and the high toxicity of certain pesticides to non-target organisms are all reasons why there is an interest in the synthesis of new pesticides of high specificity towards pests.[1–5] The Indian Institute of Chemical Technology, Hyderabad, India has been engaged for some time in the development of potential pesticides. A series of monocrotophos derivatives were synthesised and tested for their toxicity with the aim of identifying compounds of high insecticidal potency, but of lower mammalian toxicity than monocrotophos itself. A thion derivative of monocrotophos (RPR-5, Fig. 1) was found to be more toxic to target pests, *Sitophilus oryzae* L. and *Tribolium castaneum* Herbst. than monocrotophos.[6] It also had lower mammalian toxicity than monocrotophos.[6]

In view of its promising insecticidal properties, studies have been undertaken on its metabolism and toxic action. Haematological and other biochemical changes caused by this compound have been reported in rats. This thion analogue appeared to be less neurotoxic than monocrotophos in rats following short duration of exposure.[7] It was also found to deplete glutathione and inhibit glutathione S-transferase activity in hepatic and extrahepatic tissues of rat.[8]

Metabolism is one of the important determinants of

* To whom correspondence should be addressed.

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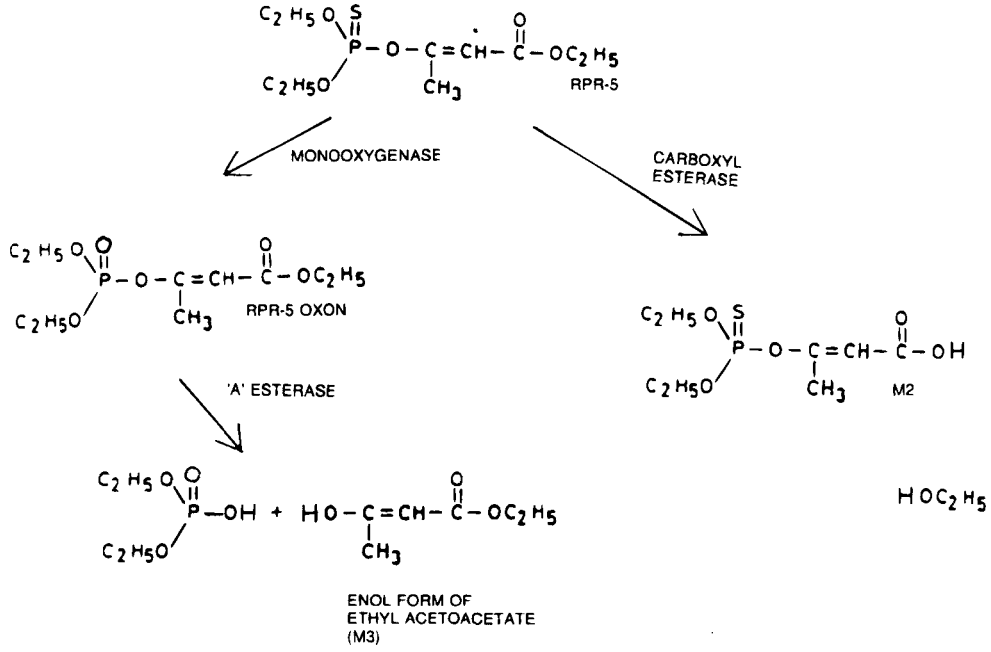

Fig. 1. Putative metabolic fate of the thion analogue of diethyl monocrotophos coded as RPR-5.

the toxic potential of compounds such as these.[9,10] The ability of thions to undergo oxidative desulfuration by hepatic microsomal monooxygenases (MO) to yield oxons and subsequent phosphorylation of the active sites of 'B-esterases' by the activated oxon is a case in point. The purpose of this study was to examine the activation of this novel organophosphate by hepatic microsomal preparations of rat and Japanese quail and the subsequent inhibition of their esterases. The inhibition of hepatic microsomal α-naphthyl acetate esterases (α-NaE) under oxidative condition was used as a measure of activation to the oxon. The in-vitro metabolism of ^{14}C-labelled RPR-5 by 11-S, microsomal and 105-S fractions of rat and quail livers was also studied to throw some light on metabolic differences between the two species which might be related to the differential toxicity of the insecticides.[3]

2 EXPERIMENTAL METHODS

2.1 Chemicals

^{14}C-Labelled RPR-5, specific activity 6 mCi mmol^{-1}, was supplied by the Indian Institute of Chemical Toxicology at Hyderabad and was purified by preparatory TLC and analysed on TLC-linear analyser for radiochemical purity (>98%). This radiolabelled preparation was diluted with unlabelled RPR-5 prior to use to give a specific activity of 2·1 uCi mg^{-1}. All the other chemicals used in the study were of Analar grade. Ethyl acetoacetate used as standard on TLC was >99% pure and was obtained from Aldrich Chemical Co. Ltd.

2.2 Rats and Japanese quails

Female Sprague-Dawley rats weighing 110–150 g were used. They were kept in polypropylene cages under a 12 h light/dark cycle, and provided with food and water *ad libitum*. Adult Japanese quails of both sexes weighing 200–300 g were obtained from Ministry of Agriculture, Food and Fisheries, Tolworth Laboratory, Surrey and kept in a large polypropylene cage under a 12 h light/dark cycle. All birds were fed on Turkey starter crumb containing wheat, soya fish meal, maize, wheat feed and liquid fat and provided with water.

2.3 Preparation of microsomes

Rats and birds were decapitated, livers quickly excised and rinsed with ice-cold potassium chloride solution (11·5 g litre^{-1}), blotted dry, weighed and placed on ice. They were then chopped with scissors and homogenised at 0–4°C for 1 min with 3 volumes of potassium chloride solution (11·5 g litre^{-1}) per gm of tissue using an MSE or Silverson homogeniser. The homogenate was centrifuged at 11 000*g* for 30 min at 4°C in an MSE Hi-spin 21 centrifuge. The 11-S supernatant was drawn off and a part of it was separated for in-vitro metabolism study while the rest was further centrifuged at 105 000*g* for 60 min at 4°C in an MSE prepspin 50 or Sorvall OTD 50B refrigerated ultracentrifuge. The microsomal pellet and 105-S supernatant so obtained were separated. The 105-S fraction was used for in-vitro metabolism and the microsomal pellet was resuspended in ice-cold potassium chloride solution (11·5 g litre^{-1}) with the help of a hand-driven Potter-Elvehem glass-

Teflon homogeniser to a concentration equivalent to 2 g liver ml^{-1}.

2.4 α-Naphthylacetate esterase assay and activation of [^{14}C]RPR-5

Four point three millilitres of reaction medium (potassium chloride 1·04, sodium dihydrogen phosphate dihydrate 8·58, sodium hydroxide 1·76, nicotinamide 0·69 g $litre^{-1}$ in water, pH 7·4) and 0·5 ml of microsomal suspension in an Erlenmeyer flask were incubated for one minute in a metabolic shaker at 37°C for rat and 42°C for quail, these representing physiological temperatures. Then [^{14}C]RPR-5 in ethanol (40 μg; 20 μl) was added to each flask. The reaction was started by addition of 0·2 ml NADPH-generating mixture (NADPH 17 mg, glucose-6-phosphate 30 mg and glucose-6-phosphate dehydrogenase 8 units per ml) whilst controls received 0·2 ml reaction medium alone. 10-μl aliquots were withdrawn after 2, 7, 10, 20 and 30 min to determine α-naphthylacetate esterase activity. At the end of incubation (30 min), substrate and metabolites were extracted as described in Section 2.5 (results not given here).

α-Naphthylacetate esterase was assayed in aliquots of the incubation medium, by the method of Gomori[11] as described by Thompson *et al.*[12] Incubation tubes contained TrisHCl buffer (25 mM; 4·9 ml; pH 7·6) and α-naphthyl acetate substrate (0·015 mM; 80 μl). The reaction was initiated by the addition of 10 μl of microsomal incubate (taken at different time intervals) and incubation was carried out at 37°C for 10 min (42°C in the case of the quail). The reaction was terminated by adding 1·0 ml of SDS (25 g $litre^{-1}$) and 1·0 ml of Fast Red ITR Salt (1 g $litre^{-1}$) in 'Triton' X-100 (25 g $litre^{-1}$). After 30 min, O.D. was measured at 530 nm on a Pye Unicam SPG-400 spectrophotometer. Samples were analysed in duplicate and inhibition of α-NaE activity at zero time, expressed as a percentage, was plotted against time (Fig. 2).

2.5 In-vitro metabolism of [^{14}C]RPR-5

In-vitro metabolism of [^{14}C]RPR-5 was studied in 11-S, microsomes and 105-S fractions of rat and quail liver by incubating ethanolic solution of RPR-5 (2 g $litre^{-1}$; 20 μl) at 37/42°C) in an Erlenmeyer flask containing 4·3 ml reaction medium, 0·5 ml 11-S fraction, microsomal suspension or 105-S fraction with and without 0·2 ml NADPH-generating mixture (no NADPH for 105-S fraction). The details of treatment are given in Tables 2 and 3. Reactions were stopped at different time intervals by extraction with diethyl ether (3 ml). Ether was selected as it was found to be an effective solvent for extraction of oxons (including malaoxon) from aqueous systems in earlier work in this laboratory; anhydrous sodium sulfate was immediately added to remove any water in the extracts. The incubation medium was then acidified with hydrochloric acid (2 M; 0·1 ml) to facilitate the extraction of any acid metabolites, followed by two further extractions with ether. The extracts were evaporated with oxygen-free nitrogen. Residues were dissolved in dry acetone and transferred to TLC plates (silica gel 60 F_{254}, 20 × 20 cm). [^{14}C]RPR-5 and unlabelled ethyl acetoacetate were applied as markers. Plates were developed in toluene + ether + acetic acid (75 + 25 + 1 by volume) and, after being air dried, RPR-5 and labelled metabolites were located using a Berthold automatic TLC linear analyser. Areas containing metabolites were carefully removed from the plate and the material from each eluted with dry acetone to recover labelled compounds. The plates were then sprayed with 2,6-dibromoquinone-4-chloroimide in cyclohexane (5 g $litre^{-1}$) and heated to enable comparison to be made with unlabelled ethyl acetoacetate standard. Ethyl acetoacetate standards appeared as brown spots and these were compared with

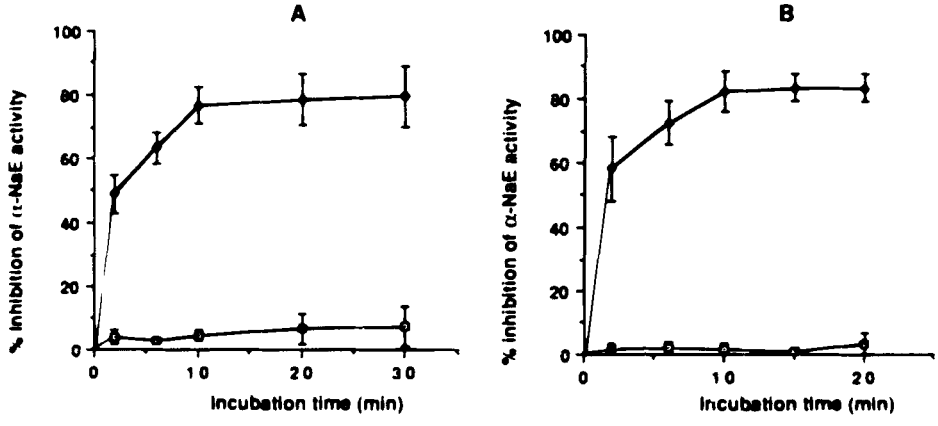

Fig. 2. Percentage inhibition of α-NaE activity by the insecticide plotted as a function of incubation time (◆) with NADPH-generating system and (□) without NADPH-generating system in hepatic microsomes of (A) rat (B) Japanese quail. Each point in the plot shows mean ± SD of six rats or five Japanese quails each carried out in duplicate.

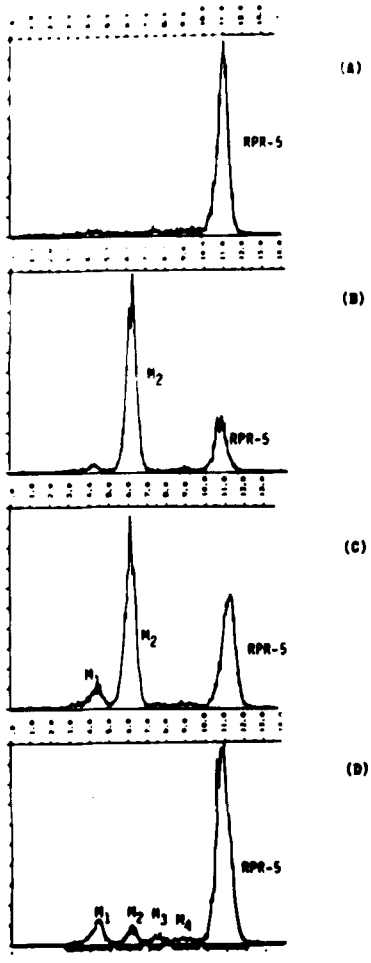

Fig. 3. Linear analyser chromatograms for TLC plates of metabolic extracts from 11-S fractions of rat liver. (A) ^{14}C-labelled insecticide standard; (B) metabolism of insecticide by 11-S fraction without NADPH-generating mixture, 30 min incubation; (C) metabolism of insecticide by 11-S fraction supplemented with NADPH-generating mixture; (D) metabolism of insecticide by 11-S preincubated with 10^{-6} M paraoxon and supplemented with NADPH-generating mixture.

adjacent areas from which labelled material had been removed. One ^{14}C-labelled metabolite (M3, Fig. 3) ran in the same position as ethyl acetoacetate.

3 RESULTS

3.1 Activation of [^{14}C]RPR-5

RPR-5 caused little inhibition of α-NaE in liver preparations in the absence of NADPH. However, when NADPH was added to the incubation mixture, inhibition occurred rapidly in both rat and quail microsomes (Fig. 2). This NADPH-dependent inhibition of α-NaE activity in rat and quail suggests that activation of RPR-5 to an 'oxon' is catalysed by microsomal monooxygenases (MO) which requires NADPH as a cofactor. Activated 'oxon' then inhibits the enzyme α-NaE. A small inhibition of α-NaE activity in the incubation medium without NADPH may be due to endogenously available NADPH (Fig. 2). After 30 min of incubation, the level of inhibition was similar in both the species.

Oxidative desulfuration of phosphorothionates by MO to generate activated 'oxon' that subsequently phosphorylates 'B-esterases' is well established.[13] Since the novel organophosphorus insecticide studied here is a thionate showing strong NADPH-dependent inhibition of α-NaE, this is presumably due to oxidative desulfuration of the insecticide.

3.2 In-vitro metabolism of [^{14}C]RPR-5

Data given in Tables 1–3 and Fig. 3 for microsomes and 11-S suggest that at least three metabolites were formed, with the possible presence of a fourth (marked M4). Their tentative identifications are given in Table 1. These were made on the following grounds. M2 was

TABLE 1
TLC Analysis of Metabolic Extracts and Standards and Determination of R_f Values of Novel Organophosphate (RPR-5) and its Metabolites[a]

Metabolite[b]	*R_f Value*	*Tentative identification*
Metabolite 1 (M1)	0·12	Oxidative product (formed by MO)
Metabolite 2 (M2)	0·26	Acid metabolite (formed by carboxylesterase)
Metabolite 3 (M3)	0·36	Ethyl acetoacetate (formed by 'A' and/or 'B' esterase)
Metabolite 4 (M4)	0·47	?Oxidative product (formed by MO)
	0·64	—
Parent Compound (RPR-5)		

[a] Solvent system used was toluene + ether + acetic acid: (75 + 25 + 1 by volume).
[b] Metabolite(s) were marked as M1, M2, M3 and M4 based on their relative mobility on silica gel TLC plate with M1 being slowest mover and M4 the fastest.

formed in rat cytosol without addition of cofactors (Table 3), and its formation was strongly inhibited by paraoxon. This suggests that it may be a product of carboxylesterase (B esterase) attack on the ethyl ester bond of RPR 5. The initial *radiolabelled* metabolite formed in this way would be the acid shown in Fig. 1. Further evidence supporting this suggestion is the relatively high polarity of M2 on TLC separation (ref. Figs 3 and 4), the fact that is not extracted by diethyl ether from aqueous media at pH 7·4 (when it will be ionised), but it is extracted at pH 2 (when it will be largely undissociated). Also, alkaline hydrolysis of [^{14}C]RPR-5 with 0·1 M alcoholic potassium hydroxide yielded a product which was extracted at pH 2 and chromatographed in the same position as M2. (Alkaline hydrolysis is expected to yield this, amongst other products). Thus, M2 is provisionally identified as the acid of RPR-5 shown in Fig. 1, suggesting esterase metabolism comparable to that which has been well established for malathion, an organophosphorus compound that also contains the ethoxycarbonyl group. M3 showed the same R_f value as an ethyl acetoacetate standard. The only clear identification of this metabolite was in rat liver 11-S preparations after paraoxon treatment and NADPH supplementation; it was not identified in quail. This would be consistent with its formation from the oxon by 'A' esterase attack. ('A' esterase activity is much higher in rat liver 11-S than in quail liver-11-S).[14] Paraoxon treatment would tend to stabilise it by inhibiting 'B' esterases which would otherwise interact with RPR oxon.

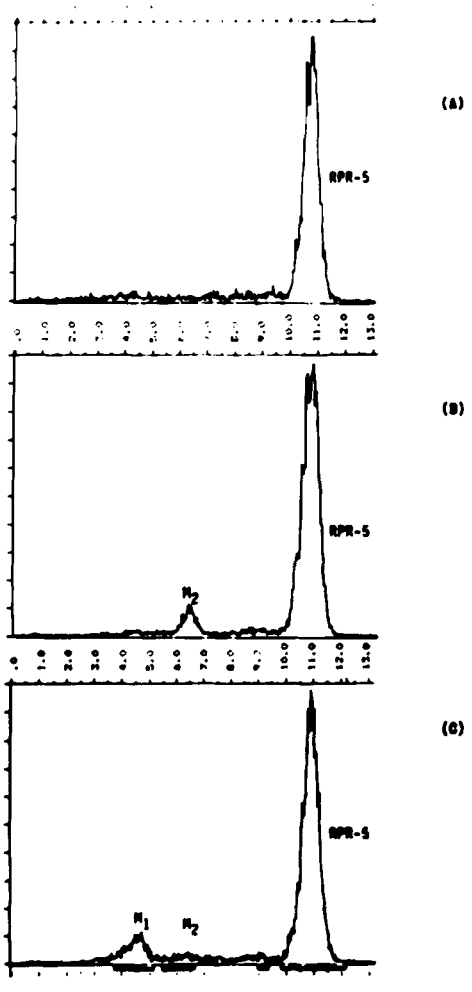

Fig. 4. Linear analyser chromatograms for TLC plates of metabolic extracts from hepatic microsomal fractions of Japanese quail. (A) ^{14}C-labelled insecticide standard; (B) metabolism of insecticide by microsomal fraction without NADPH, 2 min incubation; (C) metabolism of insecticide by microsomal fraction with NADPH, 2 min incubation.

M1 is a metabolite whose formation is clearly enhanced by NADPH supplementation of rat and quail preparations containing microsomes. It is distinctly more polar than the other metabolites, and is believed to be a product of microsomal monooxygenase attack. The possible presence of a metabolite in the position marked M4 requires further investigation, and will not be discussed further here.

In Table 2 it can be seen that with rat microsomes 58 and 73% of insecticide is metabolised, in 2 and 10 min respectively, without NADPH supplementation of the incubation medium, with the major metabolite (M2), tentatively identified as an acid. After NADPH supplementation of the incubation medium there were small reductions in both total metabolism and M4, both results expressed as percentages. The significance or otherwise of these reductions was not established. In quail, percentage metabolism by hepatic microsomes (Table 2) was substantially lower than in rat at 2 and 10 min both with and without NADPH addition. However, NADPH addition increased the percentage metabolism of insecticide with an increase in oxidative product(s) and a decrease in M2 (Fig. 4). Figure 4 shows a TLC trace of microsomal metabolism of ^{14}C-labelled RPR-5 in quail, with M2 as a major product without NADPH addition and an oxidative product M1 as a major metabolite with NADPH addition to the incubation medium. Thus the reduction of M2 production in the quail after NADPH addition may be the consequence of more RPR oxon being produced; the oxon may inhibit a carboxylesterase that generates M2. This effect was not clearly evident in the rat (see later discussion).

A comparison of ^{14}C-labelled RPR-5 metabolism by 11-S and 105-S fractions of rat and quail liver with and without paraoxon as a carboxyl esterase inhibitor (Table 3) gives some indication of the enzymes involved and their cellular location. Further, the much higher inhibition of microsomal α-NaE inhibition with NADPH supplementation than without, strongly suggests involvement of MO in the formation of activated oxon (Fig. 2). M2 formation is inhibited almost 90% in rat and 75% in quail by the preincubation of 11-S fraction with 10^{-6} M paraoxon (Table 3), which is consistent with the enzyme being a carboxylesterase.

TABLE 2
A Comparison of Metabolism by Hepatic Microsomal Fractions of Rat and Japanese Quail as Studied by TLC-Linear Analyser Chromatograms

			Rat			*Japanese quail*		
			Recovery of metabolites expressed as a percentage of total radioactivity detected on plates[ab]					
Treatment	*NADPH*	*Incubation time (min)*	*Total metabolites*	*M2*	*Others*[c]	*Total metabolites*	*M2*	*Others*[c]
Reaction medium + Microsomes	–	2	58·2 (±2·7)	57·1 (±1·9)	1·1 (±0·79)	8·7 (±0·7)	6·4 (±0·7)	2·3 (±0·8)
+ Insecticide	+	2	55·3 (±2·5)	55·3 (±2·5)	n.d.[d]	9·2 (±1·64)	1·4* (±1·0)	7·8* (±1·7)
	–	10	73·4 (±1·7)	72·7 (±2·0)	0·6 (±0·46)	11·1 (±1·52)	7·7 (±1·4)	3·4 (±0·3)
	+	10	63·4 (±5·5)	62·5 (±6·1)	0·9 (±0·69)	16·3 (±0·98)	3·4* (±1·6)	13·0* (±0·8)

[a] Data represent mean (±SD) of six rats/five Japanese quail samples, each prepared by pooling three livers.
[b] * The differences between + NADPH and – NADPH treatments were statistically significant ($P < 0·01$).
[c] One or more metabolite(s).
[d] n.d. not detected.
Note: There was 48 and 75% inhibition of α-NaE at 2 and 10 min incubation with NADPH in rats respectively. This was 58 and 72% in quail.

Formation of M2 by 105-S fraction of rat and quail is also blocked by the preincubation with 10^{-6} M paraoxon (Table 3).

In quail, M1 and other putative oxidative product(s) become the major metabolite(s) after NADPH supplementation of media containing microsomes (Table 3; Fig. 4). The availability of endogenous NADPH seems to be the reason for some oxidative product(s) formation without NADPH supplementation in the incubation medium. More interestingly, the inhibition of carboxylesterases by preincubation of 11-S fraction with paraoxon both in rat and quail (Table 3) gives an increase in oxidative product(s) presumably by reducing hydrolytic attack. It also appears from Table 3 that cytosolic carboxylesterases of rat liver are efficient vehicles for insecticide detoxication with 90% metabolism in 60 min. However, such conversion is much lower in quail (5% in 60 min).

TABLE 3
A Comparison of Metabolism by 11-S and 105 S Fractions of Rat and Japanese Quail Livers as Studied by TLC-Linear Analyser Chromatograms

			Rat			*Japanese quail*		
			Recovery of metabolites expressed as a percentage of total radioactivity detected on plates[a]					
Treatment	*NADPH*	*Incubation time (min)*	*Total metabolites*	*M2*	*Others*	*Total metabolites*	*M2*	*Others*
Reaction medium + 11-S fraction + insecticide	–	30	752	74·2	1·0	21·3	9·8	11·5
Reaction medium + 11-S fraction + insecticide	+	30	58·8	50·4	8·4	18·0	3·8	14·2
Reaction medium[b] + 11-S fraction + paraoxon + insecticide	+	30	19·4	5·9	13·5	20·0	0·9	19·1
Reaction medium + 105-S fraction + insecticide	–	30	77·4	77·4	n.d.	3·4	n.d.	3·4[c]
Reaction medium + 105-S fraction + insecticide	–	60	90·9	86·3	4·6[c]	5·6	2·5	3·1[c]
Reaction medium[b] + 105-S fraction + paraoxon	–	60	1·5	n.d.	1·5[c]	4·3	n.d.	4·3[d]

[a] Data represent observations from two different experiments done with pooled samples of six rats/five quail.
[b] Incubated for 5 min at 37°C/42°C after addition of 10^{-6} M paraoxon and then insecticide was added.
[c] Other metabolites formed by 105-S fractions in small amounts may possibly be conjugates.
[d] More than one metabolite.

4 DISCUSSION

Oxidative desulfuration of phosphorothionates by MO to generate activated oxons that subsequently phosphorylate 'B-esterases' is a well documented event.[13] The novel organophosphorus insecticide studied here is a thionate and shows strong NADPH-dependent inhibition of α-NaE. The time-dependent inhibition of carboxylesterase activity thus provides an indication of the formation of PRP-5 oxon in the rat and the quail. In both species, inhibition follows a similar pattern—rapid progression to reach 80% inhibition during the first 10 min of inhibition, with little further change up to 20 or 30 min. The reason for the lack of further inhibition after 10 min is of interest. NADPH-dependent monooxygenase activity may be expected to fall off to a considerable extent during this period. However, the plateau is rather sharply reached, and it seems more likely that a proportion of the esterase activity is resistant to inhibition by the oxon. This may just mean that certain isozymes of the carboxylesterase (a 'B'-esterase) are relatively unsusceptible to inhibition by this oxon. It is also possible that some α-NaE activity is due to a different type of esterase which is not susceptible to inhibition by oxons (e.g. a 'C'-esterase according to the classification of Aldridge).[15] In both species a principal metabolite (M2), which shows an R_f value of 0·26 on TLC, is formed in the absence of NADPH. This is thought to be an acid formed by the cleavage of the carboxylester bond by a carboxylesterase (like the conversion of malathion to its mono acid). Less of this metabolite is formed by quail microsomes than by rat microsomes. Also, addition of NADPH leads to a sharp reduction in the rate of formation of this metabolite in the quail. It would appear that this effect is due to inhibition of the carboxylesterase by the oxon generated from RPR-5. This effect is less clear in the rat than in the quail which may be due to the much higher level of A-esterase in rat microsomes[14] protecting rat carboxylesterases from inhibition by the oxon.

Earlier studies with this insecticide have shown it to be less neurotoxic than monocrotophos in rats as assessed by brain acetylcholinesterase inhibition.[7] Based on present metabolic findings it is likely that carboxylesterase hydrolysis of this insecticide provides a major route of detoxication in rats resulting in relatively little oxon formation to inhibit AChE. Monocrotophos, however, is itself an oxon which can inhibit AChE without activation. Present findings could go some way to explain the substantial difference in acute oral LD_{50} of this insecticide (30 mg kg^{-1}) and monocrotophos (9·6 mg kg^{-1}) in rat. However, the quail shows much lower rates of production of M2 than the rat, and may, therefore, be more sensitive to RPR-5 toxicity.

These results suggest that there may be a similarity between the metabolism of RPR-5 and that of malathion, viz. rapid hydrolytic detoxication by carboxylesterase attack.[16] The question is whether this detoxification is slow in insects (as in the case of malathion) and whether it makes an important contribution to the selectivity between mammals and insects.

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